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First posted online on 22 December 2017 as 10.1242/dmm.028456
Access the most recent version at http://dmm.biologists.org/lookup/doi/10.1242/dmm.028456

Suppressor of cytokine signaling 2 (SOCS2) deletion protects bone health of mice with DSS induced inflammatory bowel disease

R.Dobie[1], V.E MacRae[1], C. Pass[1] E.M. Milne[1], S.F. Ahmed[2], C. Farquharson[1*]

[1] - The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Roslin, Midlothian, Edinburgh, EH25 9RG, UK.
[2 -] School of Medicine, University of Glasgow, Royal Hospital for Children, Govan Road, Glasgow, G51 4TF, UK.

Keywords: SOCS2, Inflammatory bowel disease, growth hormone, bone

*Address for Correspondence

Professor Colin Farquharson,
The Roslin Institute and Royal (Dick) School of Veterinary Studies,
University of Edinburgh,
Easter Bush,
Roslin,
Midlothian.
EH25 9RG.
UK
Tel 0131 651 9176
Fax 0131 651 9105

Email colin.farquharson@roslin.ed.ac.uk

Summary Statement – Utilising a mouse model of inflammatory bowel disease this article provides an improved understanding of the relative effects of GH/IGF-1 on bone health in experimental colitis.

Abstract

Individuals with inflammatory bowel disease (IBD) often present with poor bone health. The development of targeted therapies for this bone loss requires a fuller understanding of the underlying cellular mechanisms. Although bone loss in IBD is multifactorial the altered sensitivity and secretion of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in IBD is understood to be a critical contributing mechanism. The expression of suppressor of cytokine signaling 2 (SOCS2), a well-established negative regulator of GH signaling, is stimulated by pro-inflammatory cytokines. Therefore, it is likely that SOCS2 expression represents a critical mediator through which pro-inflammatory cytokines inhibit GH/IGF-1 signaling and decrease bone quality in IBD.

Utilising the DSS model of colitis we have revealed that endogenously elevated GH function in the *Socs2*$^{-/-}$ mouse protects the skeleton from osteopenia. Micro-computed tomography assessment of DSS treated wild-type mice revealed a worsened trabecular architecture compared to control mice. Specifically, DSS treated WT mice had significantly decreased bone volume (BV/TV) (41%; $p<0.05$), trabecular thickness (16%; $p<0.05$), trabecular number (30%; $p<0.05$), and a resulting increase in trabecular separation (19%; $p<0.05$). In comparison, the trabecular bone of *Socs2* deficient mice was partially protected from the adverse effects of DSS. The reduction in a number of parameters including BV/TV (21%; $p<0.05$) was less, and no changes were observed in trabecular thickness or separation. This protected phenotype was unlikely to be a consequence of improved mucosal health in the DSS treated Socs2$^{-/-}$ mice but rather a result of unregulated GH signaling directly on bone.

These studies indicate that the absence of SOCS2 is protective against bone loss typical of IBD. This study also provides an improved understanding of the relative effects of GH/IGF-1 on bone health in experimental colitis, information that is essential before these drugs are explored as bone protective agents in children and adults with IBD.

Introduction

Inflammatory bowel disease (IBD), which includes the chronic intestinal disorders, Crohn's disease (CD) and ulcerative colitis (UC), is considered to result from an inappropriate inflammatory response to intestinal microbes in genetically susceptible hosts (Abraham and Cho, 2009). They are life-long conditions with a prevalence of 5 per 1000 people and annual health care costs exceeding $1.7 billion in the United States (Sandler *et al*., 2002, Lakatos *et al*., 2006). In addition, to the well-recognized gut inflammation associated with IBD, both children and adults have poor bone health and are at increased risk of fractures which cannot solely be attributed to exogenous glucocorticoid exposure (Thearle *et al*., 2000; van Staa *et al*., 2003; Wong *et al.,* 2016)). The relative risk of fracture in adults is 40% higher than normal (Compston *et al*., 1987) and a vertebral fracture may be present in 10% of affected people (Laakso *et al*., 2012; Wong *et al*., 2014). Recent studies in children have also shown that trabecular bone density, which was reduced at diagnosis, showed inadequate improvement despite control of the underlying inflammation (Dubner *et al*., 2009). These patients also have abnormal bone geometry with thinner and smaller bones (Dubner *et al.,*2009; Tsampalieros *et al.,*2013). Furthermore, peak bone mass is compromised in childhood onset IBD, despite adequate control of disease and progression through puberty (Laakso *et al*., 2014).

The etiology of bone loss in IBD is multifactorial, with risk factors including steroid medication, poor nutrient intake/absorption and the underlying inflammatory state (Bernstein *et al*., 2013). Central to the inflammatory response in IBD is the over production by T lymphocytes and macrophages of various pro-inflammatory cytokines such as interleukin (IL) -1, -2, -6, -8, and tumor necrosis factor (TNF)-α (Ali *et al*., 2009). IL-6 has been identified as the predominant cytokine mediating the bone abnormalities, and genetic variations in *IL-6* correlate well with the clinical course of IBD and the extent of bone loss (Schulte *et al*., 2000). Although pro-inflammatory cytokines are known to promote bone loss directly, they also lead to altered sensitivity and secretion of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in IBD which may be another critical mechanism leading to osteoporosis (Wong *et al*., 2010).

GH and IGF-1 are recognized stimulators of bone mass (Giustina *et al*., 2008). Transgenic mice over expressing *Igf1* in osteoblasts exhibit increased trabecular bone, whereas *Igf1* null mutants exhibit reduced cortical bone (Liu *et al*., 1993; Zhao *et al*., 2000). $GHR^{-/-}$ mice have reduced bone turnover, cross-sectional cortical bone area, and cortical growth, whereas GH overexpression results in increased bone cortical area (Sjogren *et al.,* 2000; Sims *et al*., 2000; Eckstein *et al*., 2004). Skeletal

manifestations are also observed in humans with GH deficiency who present with low bone turnover osteoporosis (Doga *et al*., 2005) which can be ameliorated by rhGH replacement therapy (Hansen *et al.,*1996). The therapeutic benefits of rhGH administration on bone health of IBD patients remain unknown. Nevertheless, given that both rhGH and rhIGF-1 are available as therapeutic drugs, there is potential for these anabolic drugs to improve bone health in people with IBD.

Suppressor of cytokine signaling 2 (SOCS2) regulates GH signalling during normal growth and development. This physiological role is not restricted to bone. This function is most clearly observed in the *Socs2* KO mouse where a number of organs are increased in size and these include liver, heart, lungs, bladder (Metcalf *et al*., Nature 2000). In another study SOCS2 has been shown to limit intestinal growth (Michaylira et al., 2005). SOCS2 is also induced by a subset of pro-inflammatory cytokines *e.g.* IL-6, IL-1b and TNFα which are elevated in IBD and mediate the inflammatory response (Starr *et al*., Nature 1997; Greenhalgh & Hilton Biol 2001; Rico-Bautista *et al.,* 2006; Sanchez-Munoz *et al*,. 2008; MacRae *et al.,* 2009). However, this protection against inflammation may come at the expense of poor bone health through the induction of SOCS2 and the inhibition of GH signalling (Denson *et al*., 2003; Shi *et al*., 2004).

SOCS2 is expressed by many cells, including osteoblasts, and inhibits GH signaling via inhibition of the JAK/STAT intracellular signaling pathway (Greenhalgh *et al*., 2002; Flores-Morales *et al*., 2006; Michaylira *et al*., 2006, Pass et al., 2012, Dobie *et al*., 2014). *Socs2* deficient mice, have unrepressed GH signaling and present with an overgrowth and high bone mass phenotype (Metcalf *et al*., 2000; MacRae *et al*., 2009; Dobie *et al*., 2014). These data emphasize the critical role for SOCS2 in controlling GH's anabolic effects on bone. Therefore, it is possible that increased osteoblast SOCS2 expression represents a critical mediator through which pro-inflammatory cytokines inhibit GH/IGF-1 signaling and decrease bone mass and quality in patients with IBD.

To directly examine this, we studied bones from a mouse model of IBD using dextran sodium sulfate (DSS) induced colitis and investigated the potential of ablating the expression of *Socs2* to endogenously elevate GH and IGF-1 function, and correct the bone loss observed in murine colitis. This study is the first-ever in an IBD animal model to investigate whether GH and IGF-1 have bone protective effects. Furthermore, data from this innovative approach will help inform the design of novel therapies that are directed specifically at the mechanism of insult which leads to poor bone health in children and adults with IBD.

Results

Effect of DSS treatment on body-weight of WT and Socs2$^{-/-}$ mice.

To investigate the effects of DSS induced colitis on bone development and the possible role of SOCS2 in mediating bone loss, WT and *Socs2*$^{-/-}$ mice were treated with 3% DSS for 4 days (Fig. 1). The dose and duration of DSS treatment was based on results from a dosing experiment where mice were given DSS at varying concentrations for 4-5 days (Fig. S1) During the DSS treatment period (0-4 days) no weight loss was observed in WT or *Socs2*$^{-/-}$ mice (Fig. 1). Independent of genotype, mice exhibited a rapid weight loss from day 4 (Fig. 1). There was no significant difference in maximum weight loss observed between WT (15%) and *Socs2*$^{-/-}$ (13%) mice (Fig. S1B). Following the period of weight loss, all DSS treated mice continued to gain weight to the end of the study (day 18) (Fig. 1). Pair fed control mice did not show any significant alterations in body weight throughout the experiment (Fig. 1). During the period of DSS treatment, water intake was similar in WT and *Socs2*$^{-/-}$ mice. Also, food intake during DSS intake and the periods of weight loss and recovery were similar (data not shown).

In terms of weight loss, WT mice showed high individual variation in susceptibility to DSS (Fig. S1C). Maximum body weight loss ranged from 6% to 21% in WT mice treated with 3% DSS. In contrast, body weight loss observed in *Socs2*$^{-/-}$ mice treated with 3% DSS was more uniform, ranging from 11% to 15% before recovering (Fig. S1C).

Effect of DSS treatment on longitudinal growth of WT and Socs2$^{-/-}$ mice.

Socs2$^{-/-}$ mice are characterised by their overgrowth phenotype (Greenhalgh *et al*., 2002). In agreement with this, the untreated *Socs2*$^{-/-}$ mice at the end of this study (day 18) were 39% ($p<0.001$) heavier than untreated WT mice (Table 1). Also, tibia (6%; $p<0.01$), and femur (8%; $p<0.01$) length as well as nose to rump length (13%; $p<0.001$) were all greater in untreated *Socs2*$^{-/-}$ mice compared to WT mice (Table 1). DSS treatment had no effect on the body-weight of WT and *Socs2*$^{-/-}$ mice at the end of the study (Table 1). Similarly, DSS treatment did not result in altered nose to rump length, tibia length, or femur length of WT or *Socs2*$^{-/-}$ mice (Table 1).

Colon pathology of DSS treated mice

To assess the effects of DSS on mucosal integrity, detailed histological analysis was performed on the colon from control and DSS treated WT and *Socs2*$^{-/-}$ mice. Histology scores were minimal in the non-DSS treated (control) mice and furthermore, there were no notable differences in colon diameter, morphological differences or differences in histological scoring between the non-DSS treated (control) WT and *Socs2*$^{-/-}$ mice (Fig.2A, B&C). This infers that the ablation of *Socs2* alone had no

obvious effects on colon morphology. In contrast, histological analysis of the colon from DSS treated WT and *Socs2*$^{-/-}$ mice, revealed extensive levels of inflammation. DSS treated mice were characterised as having signs of both acute and chronic inflammation throughout their colon (Fig. 2A). Signs of acute inflammation included infiltration of neutrophils into the lamina propria and submucosa (Fig. 2A), and epithelial degeneration (Fig. S2). In a number of sections there were also signs of crypt loss (Fig. S2). In addition to acute inflammation, there were also high levels of mononuclear leucocytes (macrophages, lymphocytes and plasma cells) (Fig. 2A & Fig. S2), and transmural inflammation (Fig. S2), which are recognised markers of chronic inflammation.

Scores for inflammation severity and extent of inflammation were significantly increased in DSS treated mice (Fig. 2B). However, the histological mean scores for inflammation severity (WT: 6.8, *Socs2*$^{-/-}$: 5.2) and extent of inflammation (WT: 7.4, *Socs2*$^{-/-}$: 6.0) following DSS treatment were not significantly different between genotypes, nor was colon diameter (Fig. 2B&C). In contrast, there was a significant difference in crypt damage/regeneration mean score between DSS treated WT and DSS treated *Socs2*$^{-/-}$ mice ($p<0.05$) (Fig. 2B). This difference in crypt damage/regeneration between genotypes following DSS treatment was not sufficient however to alter total pathology scores indicating that the absence of *Socs2* was unable to confer overall protection against DSS induced gut inflammation (Fig. 2B). Although not part of the scoring system, the number of goblet cells in the mucosa of the colon was reduced in areas of loss of the epithelium as a result of the inflammatory response, and in areas of crypt damage and regeneration in DSS-treated WT and *Socs2* deficient mice.

Systemic IGF-1 levels in DSS treated mice

GH resistance has been associated with IBD, and is characterized by a decrease in systemic IGF-1 levels (Ballinger *et al.,* 2000; Katsanos *et al.,* 2001). Analysis of serum from DSS treated animals revealed that IGF-1 levels were similar in control and DSS treated WT (WT control 263.8 ± 6.2ng/ml; DSS treated WT 243.6 ± 10.8ng/ml) and *Socs2*$^{-/-}$ mice (*Socs2*$^{-/-}$ control 264 ± 9.8ng/ml; DSS treated *Socs2*$^{-/-}$ 276.9 ± 17.6ng/ml).

Socs transcript levels in bones of DSS treated mice

Socs1, 2, and *3* transcript levels were measured in bone samples from control and DSS treated WT mice. *Socs2* (2.7 fold; $p<0.05$) and *Socs3* (4.1 fold; $p<0.05$) levels were higher in DSS treated mice, but no significant difference was observed in *Socs1* levels (Fig. 3).

Bone phenotype of DSS treated mice

DSS induced colitis has previously been shown to have detrimental effects on bone quality, in both juvenile (4 week old) and young adult (10 week old) mice (Hamdani *et al.,* 2008; Harris *et al.,* 2009). In accordance with these studies, DSS treated WT mice showed worsened trabecular architecture compared to control mice as demonstrated by µCT (Fig 4). Specifically, DSS treated WT mice had significantly decreased bone volume/tissue volume (BV/TV) (41%; $p<0.05$), trabecular thickness (16%; $p<0.05$), and trabecular number (30%; $p<0.05$), and a resulting increase in trabecular separation (19%; $p<0.05$) (Fig. 4). The increase in trabecular pattern factor (40%; $p<0.05$) and structural model index (14%; $p<0.05$) indicates a more disconnected 'rod-like' trabecular structure, which are associated with reduced trabecular micro-architecture quality (Hildebrand and Rüegsegger, 1997). In comparison, DSS treated *Socs2*$^{-/-}$ mice showed a much less severe alteration in trabecular architecture compared to control mice (Fig. 4). Specifically, the reduction in BV/TV (21%; $p<0.05$) and trabecular number (14%; $p<0.05$), and increase trabecular pattern factor (19%; $p<0.05$) and structural model index (6%; $p<0.05$) were between 50%-55% less severe than that noted in DSS treated WT mice (Fig 4B). Furthermore, trabecular thickness and trabecular separation, in contrast to DSS treated WT mice, were not significantly different in DSS treated *Socs2*$^{-/-}$ mice compared to controls (Fig 4B). These striking data indicate that the level of bone loss in DSS colitis is influenced by *Socs2* expression and furthermore suggest that the absence of SOCS2 is partially protective against bone loss typical of IBD.

Analysis of cortical bone in control and DSS treated WT and *Socs2* deficient mice revealed no change in any parameters following DSS treatment (Table 2). Nonetheless, the anabolic bone phenotype (characterized by increased cortical tissue area ($p<0.05$), bone area ($p<0.05$), cortical thickness ($p<0.05$), and marrow area ($p<0.05$) was apparent in untreated *Socs2*$^{-/-}$ mice (Table 2).

Discussion

Murine models of IBD have been used extensively to better understand disease etiology and therapeutic opportunities. In this study we used the DSS-induced colitis mouse model which has been extensively validated by others to induce acute and chronic forms of IBD (Wirtz *et al.,* 2007; De Roberts *et al.,* 2011; Kanneganti *et al.,* 2011). This model has also been used to study the link between IBD and the risk of colorectal cancer (Bollrath et al., 2009). The strength of this model is that histological changes seen can include a wide range of features that are associated with chronic forms of IBD in man (Perse and Cerar, 2012). DSS induced colitis is the result of deterioration of the

epithelial barrier through an increase in epithelial cell apoptosis and a decrease in proliferation (Araki *et al.,*2010). This deterioration allows for the influx of antigens and micro-organisms; prompting an increased expression of inflammatory mediators (*e.g.* TNF-α, IL-1β, IFN-γ, IL-10, IL-6 and IL-12) which drive the pathogenesis of DSS-induced colitis (Tlaskalova-Hogenova *et al.,* 2005; Perse and Cerar, 2012).

DSS induced acute colitis has previously been shown to result in reduced bone health in juvenile and young adult mice. During active disease and early recovery in juvenile mice, both the trabecular and cortical bone compartments are adversely affected whereas DSS-treated older mice exhibit reduced femoral bone mass and altered micro architecture. No changes were observed in cortical bone indices (Hamdani *et al.,* 2008; Harris *et al.,* 2009).

In the present study we found a severe trabecular bone loss phenotype in WT mice treated with DSS, but no changes in cortical bone parameters. These data are consistent with previous reports in similarly aged adult mice (Hamdani *et al.,* 2008). The trabecular bone phenotype was characterized by lowered BV/TV, trabecular thickness, trabecular number and increased trabecular separation. Taken together, these studies suggest that there is an age-specific effect of DSS induced IBD on bone loss in mice. During early puberty (3 to 5 weeks) there is extensive radial bone expansion and this may explain why the loss of cortical bone is restricted to juvenile mice (Callewaert *et al.,* 2010). DSS treatment did not result in altered nose to rump length, tibia length, or femur length of WT or *Socs2*$^{-/-}$ mice. This is contrast to a previous study which reported decreased bone length and growth plate thickness and altered chondrocyte marker gene expression in young mice (four-week-old) treated with DSS (Harris *et al.,* 2009). Whilst bone length was fully restored at the end of the study these data are in accord with human studies that show IBD can stunt growth and diminish final height (Burnham 2004; Sylvester *et al.*, 2007). It is likely that we did not see a reduction in bone length in this study due to the older mice (8-9 weeks of age) studied by which time the rapid phase of linear bone growth was completed.

SOCS2 expression is induced by pro-inflammatory cytokines and is a recognized inhibitor of GH signaling (Denson *et al.,* 2003). Furthermore, *Socs2* deficient mice have uninhibited GH signaling, leading to increased body-weight and a high bone mass phenotype (Metcalf *et al.,* 2002; MacRae *et al.,* 2009; Dobie *et al.,* 2014). GH is a recognized stimulator of bone mass (Giustina *et al.,*2008). Global GH overexpression results in increased cortical cross-sectional area (Eckstein et al., 2004). Increased cortical area and bone strength is also observed in human growth hormone transgenic mice, where expression of GH is directed specifically to osteoblasts with minimal systemic overproduction (Tseng *et al*., 1995) Conversely, mice carrying mutations of the GH receptor have

reduced femoral width and tibial cortical thickness, and an associated reduction in periosteal bone growth (Sims *et al.,* 2000). Skeletal manifestations are also observed in humans with GH-deficiency who present with low bone turnover osteoporosis, leading to increased fracture risk (Doga *et al.,* 2014). Using an osteoblast culture model we have previously shown that GH's promotion of STAT5 phosphorylation and nuclear translocation is enhanced in *Socs2* deficient osteoblasts, whereas *Socs2* overexpression in osteoblasts blunts GH's effect (Dobie *et al.,* 2014). These observations stress the key role for SOCS2 in controlling GH's anabolic skeletal effects and are extended by data from this present study which reveals that *Socs2* expression is higher in bone samples from DSS treated WT mice. This increased *Socs2* expression, a probable consequence of higher levels of circulating pro-inflammatory cytokines, is likely to repress GH signaling and contribute to the poor bone health noted in colitic (DSS treated) WT mice.

In this study, we examined the hypothesis that elevated GH signaling in mice deficient in SOCS2 protein would offer protection against bone loss in a murine model of IBD. Such protection was observed in trabecular bone where in the absence of SOCS2, DSS treatment resulted less severe changes to the trabecular bone architecture. This underscores the importance of *Socs2* ablation in protecting against bone loss in DSS induced IBD. Complete protection of the skeleton was not observed however, and this is likely due to other cellular mechanisms including, increased osteoclastic resorption, direct (non-GH mediated) effects of pro-inflammatory cytokines on osteoblast differentiation/function and diminished GH signaling through elevated *Socs3* levels. The effects of ablating both *Socs2* and *Socs3* in the DSS model would be of interest as it may offer enhanced protection to the skeleton. Previous studies have shown that the removal of GH signaling deletes the $Socs2^{-/-}$ phenotype which includes the normalisation of body weight and bone length (Greenhalgh *et al*., 2002, 2005). Therefore, it is likely that mice in which both *Socs2* and GH signaling are silenced there would be no protection from DSS induced bone loss. The gathering of such evidence would provide functional evidence for GH's pivotal role in protecting bone health in this animal model of IBD. Furthermore, it is also worth noting that reduced expression of SOCS2 has been associated with increased cancer risk which may be a consequence of increased GH signalling (Hendriksen et al., 2006). Therefore, if GH treatment or novel therapies to target the SOCS2 protein are to be considered as bone protective agents in children and adults with IBD then the potential cancer risk has to be carefully considered.

Histologic scoring in the present study was carried out using a validated scoring scheme, allowing an in depth assessment of the mucosal integrity (Dieleman *et al.,* 1998; Williams *et al.,* 2001). Acute and chronic colitis are characterised by distinct pathological changes to the colon. Acute inflammation is associated with an influx of neutrophils into the lamina propria, and in some cases epithelial degeneration. Chronic inflammation on the other hand is associated with mononuclear leukocyte infiltration, crypt architectural disarray, and crypt regeneration (Melgar *et al.,* 2005; Perse & Cerar 2012). In this study, histological analysis revealed signs of both acute and chronic inflammation in the colons of all DSS treated mice, suggesting that the current experimental design was sufficient to induce chronic inflammation. Previous reports have shown that acute inflammation can progress to chronic in C57BL/6 mice following a single treatment of DSS (Melgar *et al.,* 2005). Importantly, little difference was observed between the pathology scores of colons from DSS treated WT and *Socs2*$^{-/-}$ mice. This pathological assessment suggests that the absence of SOCS2 does not appear to protect against the deterioration of mucosal integrity in DSS experimental colitis and therefore the improved bone health noted in the DSS treated *Socs2* deficient mice is unlikely to be solely attributed to improved disease status. However, it is recognised that further studies to quantify cell apoptosis, proliferation and stem cell number within the colon would allow us to make a more definitive judgement on colon health in the DSS treated WT and SOCS2 deficient mice.

Intriguingly, there have been reports that increased GH activity promotes mucosal repair during IBD associated inflammation. A small clinical trial of patients with active CD reported that therapy with recombinant human GH therapy improved the CD activity index, and decreased the need for other medication (Slonim *et al.,* 2000). Previous studies have also reported that in mice with a mutated gp130 receptor (gp130Y757F mice) there is protection to the colon from the damaging effects of DSS (Bollrath et al., 2009). It is possible that in the gp130Y757F mice in which SOCS3 cannot inhibit JAK/STAT signaling there is a protective pathway(s), involving increased STAT3 activation that may not be active in SOCS2 deficiency.

Furthermore, GH transgenic mice display a similar extent of colon pathology during the onset of inflammation compared to WT mice, but show improved mucosal repair over an extended time period (Williams *et al.,* 2001). Increased mucosal repair in the GH transgenic mice may be a result of increased systemic IGF-1, which has been reported to partially attenuate colonic damage in the DSS rat model (Mathews *et al.,*1988; Howarth *et al.,*1998). Whilst we noted subtle improvements in the crypt damage/regeneration score of DSS treated *Socs2*$^{-/-}$ mice this is unlikely to be related to systemic IGF-1 status of these mice, which were found to be normal. However, it must be noted that

gut IGF-1 levels were not measured in these mice. Therefore the possibility of local upregulation of IGF-1 in response to unregulated GH signalling in *Socs2*$^{-/-}$ mice cannot be ruled out. Despite the slight improvement in the crypt damage/regeneration score of DSS treated *Socs2*$^{-/-}$ mice, their total pathology score was similar to DSS treated WT mice. It is however prudent to note that in our studies systemic IGF-1 levels were measured at the end of the experiment when the DSS treated animals presented with severe inflammation of the colon, but had recovered their body-weight. Previous studies have shown decreased systemic IGF-1 levels with active disease which returned to normal with recovery (Harris *et al.,* 2009). Further studies are required to understand fully the role of systemic IGF-1 in the skeletal response to experimental IBD. In keeping with previous research, systemic IGF-1 levels in WT and *Socs2*$^{-/-}$ control animals were comparable, further confirming the importance of the direct anabolic effects of GH on bone (Metcalf *et al.,* 2000; Lorentzon *et al.,* 2005; MacRae *et al.,* 2009; Dobie *et al*., 2014, Dobie *et al*., 2015).

The negative effects of increased *Socs2* expression in inflammatory conditions may not be restricted to the skeleton. In rodent models of chronic kidney disease there is increased *Socs2* gene expression in liver and muscle which may contribute to impaired phosphorylation and nuclear translocation of GH-activated STAT proteins and the development of GH resistance (Schaefer *et al.,* 2001, Sun *et al.,* 2004; Mak and Cheung 2007, Cheung *et al.,* 2008). Intervention strategies to reduce uremic cachexia are associated with amelioration of the uremia-associated increase in *Socs2* expression (Cheung *et al.,*2008). Recent data has also shown that *Socs2* deletion protects against streptozotocin-induced type 1 diabetes in adult male mice possibly through increased β-cell hypersensitivity to GH (Alkharusi *et al.,*2015). It is therefore likely that SOCS2 signaling represents a generic critical pathway through which pro-inflammatory cytokines alter both GH/IGF-1 signaling and cellular function (Ahmed and Farquharson, 2010; Farquharson and Ahmed, 2013).

In conclusion, these studies suggest that the absence of SOCS2 is protective against bone loss typical of IBD and are consistent with the premise that increased osteoblast SOCS2 expression represents a critical mediator through which pro-inflammatory cytokines inhibit GH signaling and decrease osteoblast function and bone accrual. This study also provides an improved understanding of the relative effects of GH/IGF-1 on bone health in experimental colitis and is consistent with data reporting the beneficial effects of GH on bone in conditions such as juvenile idiopathic arthritis (Bechtold *et al.* 2009). This accumulation of information is essential before these drugs are explored as bone protective agents in children and adults with IBD.

Materials and Methods

Mice

It is recognised that different genetic strains of mice respond differently to DSS (Melgar *et al*., 2005; Perse and Cerar 2012). Therefore, we backcrossed our original SOCS2 knockout (KO) mice (76.0% C57BL/6) (MacRae *et al.,* 2009) with pure C57BL/6 mice a further six-times to establish our SOCS2 KO mice and litter mate wild-type (WT) controls on a pure (>99.0%) C57BL/6 background strain. These mice were used in all studies reported. For genotyping, ear biopsied DNA was analysed by PCR for SOCS2 (WT) or the neocassette (*Socs2*$^{-/-}$) using the following primer sequences: SOCS2 - Forward (5'-3') TGTTTGACTGAGCTCGCGC, Reverse (5'-3') CAACTTTAGTGTCTTGGATCT and Neo - Forward (5'-3') ACCCTGCACACTCTCGTTTTG Reverse (5'-3') CCTCGACTAAACACATGTAAAGC. Primers were obtained from Eurofins MWG Operon, London, UK. All animal experiments were approved by Roslin Institute's Animal Users Committee, and the animals were maintained in accordance with Home Office (UK) guidelines for the care and use of laboratory animals.

Establishment of acute DSS induced colitis model

Male WT and *Socs2* KO mice (6 mice per group), 8-9 weeks of age, received 3.0% DSS (molecular weight ~40000kD; Sigma Aldrich, UK) in their drinking water (tap water). They were given DSS treated water ad lib for 4 days, following which they received normal tap water for a 14 day recovery period. Control (non DSS treated) male WT and *Socs2* KO mice (6 mice per group) were offered normal tap water for the duration of the study. The health status of the DSS treated mice was scored daily, with particular attention paid to their coat condition, mobility, blood in stools and eye clarity. Body weights of all mice were recorded daily. To establish the weight loss that was due to inflammation and not lowered food intake, the mice were pair-fed. The quantity of food consumed daily (fed *ad lib*.) by the DSS treated mice was weighed and then provided to control animals (who received no DSS) the following day (Ballinger *et al*., 2000). All mice were housed individually to allow accurate measurement of food and water intake and health status. Eighteen-days after the initiation of the studies the experiment was stopped, blood collected for serum analysis, and the long bones and the colon dissected.

Colon histology

The colon was dissected from WT and *Socs2*$^{-/-}$ mice ± DSS treatment and fixed and stored in 4% paraformaldehyde. Each colon was divided into 5 transverse segments including proximal to distal portions. Tissue processing, wax embedding, sectioning (5µm thick) and H&E staining were done following routine procedures. Colon pathology was graded blind on sections from all 5 segments of each mouse using an established histological grading scheme (Dieleman *et al*., 1998). Segments of colon were assessed separately for inflammation. Scores from all five segments were averaged to provide an overall pathology score. Colon diameters were measured on the H&E sections. Using image analysis software the diameter of each transverse segment was measured twice and averaged.

Microcomputed tomography

To evaluate trabecular architecture and cortical bone geometry of the tibia from control and DSS treated mice we used a µCT system (Skyscan 1172 X-Ray microtomograph, Bruker Corporation, Kontich, Belgium) as described previously (Dobie *et al.,*2014). In brief, high-resolution scans with an isotropic voxel size of 5 µm (trabecular bone) or 10 µm (cortical bone) were acquired (60 kV, 0.5 mm aluminium filter, 0.6° rotation angle). Two images were averaged at each rotation angle. Scan reconstruction was done using NRecon software (Bruker). A 1 mm section of the metaphysis was taken for the analysis of trabecular bone, using the base of the growth plate as a standard reference point. A 500 µm section of the mid-shaft was taken for the analysis of cortical bone, using the articulation with the fibula as a standard reference point. CTAn software (Bruker) was used to analyse the appropriate parameters (Bouxsein *et al.,* 2010).

Serum IGF-1 ELISA

Blood obtained by cardiac puncture was stored in serum tubes (Greiner Bio-One, Gloucestershire, UK) on ice for over 30mins to allow for clotting. Following centrifugation for 10 mins at 1000g, supernatant was removed, aliquoted, and stored at -80°C. IGF-1 levels were assessed by ELISA (Quantikine, R&D Systems, Minneapolis, USA) according to manufacturer's instructions.

RNA extraction and RT-qPCR analysis

Left femur were dissected from WT and *Socs2*$^{-/-}$ mice ± DSS treatment. The femurs had both epiphyses removed and the marrow was spun out by centrifugation and discarded. The bone samples were snap frozen in liquid nitrogen and stored at -80°C. Bone samples were ground using a

mortar and pestle, and homogenised by a hand held homogeniser in QIAzol Lysis Reagent. RNA from extracted using an RNeasy Lipid Tissue Kit (Qiagen Ltd, Manchester UK) following manufacturer's protocol. RNA content was measured by absorbance at 260 nm, and quality by 260/280 ratios. RNA was stored at -80°C. RT was carried out as described previously (Newton *et al* 2014). RT-qPCR was performed using the SYBR green (Roche detection method on a Stratagene Mx3000P real-time qPCR machine with MxPro software (Stratagene, Santa Clara, CA, USA). Relative gene expression was calculated using the DDCt method (Livak and Schmittgen, 2001). Each cDNA sample was normalized to housekeeping gene *gapdh* (Primer Design, Southampton, UK). Reactions were performed with gene of interest primers - *Socs1* (For– TCCGATTACCGGCGCATCACG; Rev– CTCCAGCAGCTCGAAAAGGCA), *Socs2* (For – TGGCTGCTCAAGATCAAATG; Rev- TGTCCTCCTGGAAATGGAAG) and *Socs3* (For- GAGTACCCCCAAGAGAGCTTACTA; Rev- CTCCTTAAAGTGGAGCATCATACTG) (MWG Eurofins).

Statistical analysis

All measurements and analysis were done blinded to the researcher. All statistical analysis was completed using GraphPad Prism. Final measurements, IGF-1 ELISA, and histology scoring data were analysed using a two-tailed unpaired t-test with Welch's correction (equal standard deviations not assumed) or suitable nonparametric test (Mann-Whitney) if the data were not normally distributed. Due to small sample size transcript and µCT data were analysed using a nonparametric test (Mann-Whitney). Data presented as mean ± SEM.

Acknowledgements.

The authors are grateful for the technical assistance of Elaine Seawright, and Darren Smith and Alex Robertson of the Small Animal unit towards the completion of these studies. This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) UK and Ipsen (UK) through a CASE studentship award (RD), and Institute Strategic Programme Grant Funding from the BBSRC (BB/J004316/1)

Competing interest's statement

The authors declare no conflicts of interest

Author contributions

CF and SFA supervised the project; CF,RD, VEM and SFA conceived and designed the experiments; CF and RD wrote the manuscript; RD, CP and EMM, performed the experiments and analyzed the data; CP, EMM, VEM and SFA helped to prepare the manuscript. All authors read, discussed and edited the manuscript.

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Figures

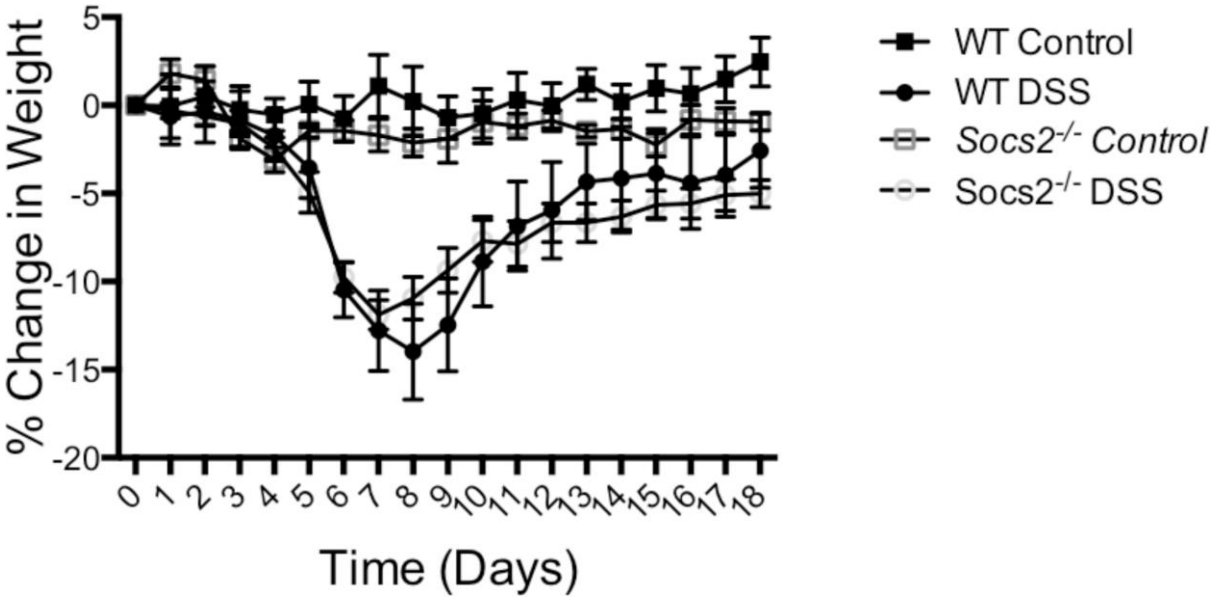


Figure 1. **Body weight changes of WT and *Socs2*$^{-/-}$ mice treated with DSS followed by recovery period.** Percentage change in body weight of WT and *Socs2*$^{-/-}$ mice treated with 3% DSS for 4 days. Data presented as mean ± SEM (n=6).

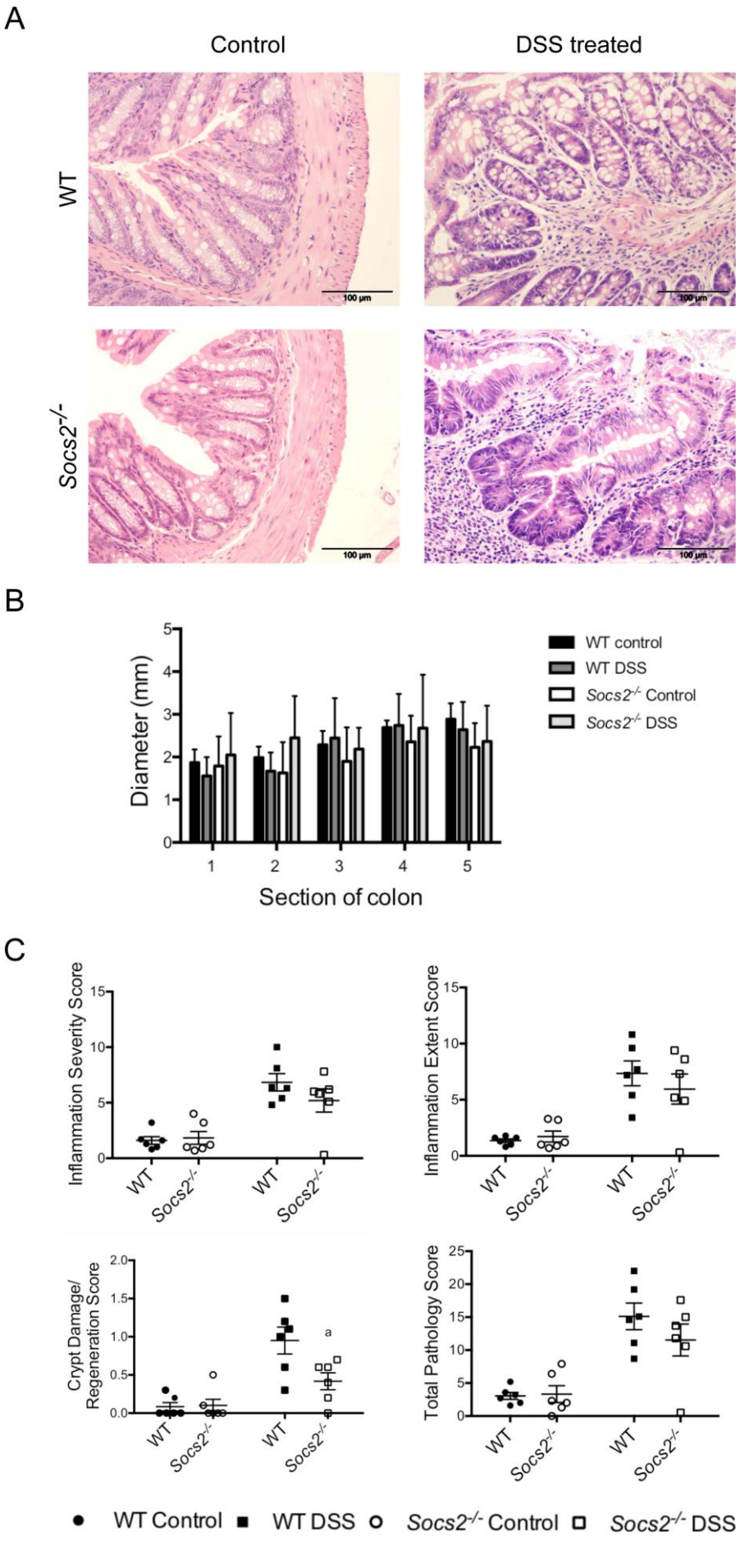


Figure 2. **Colon pathology of DSS treated WT and *Socs2*$^{-/-}$ mice.** **A.** Representative H&E stained sections of colon from control and DSS treated WT and *Socs2*$^{-/-}$ mice. **B.** Diameter of DSS treated, and control WT and *Socs2*$^{-/-}$ colons. Numbers 1-5 represent difference segments along the colon. **C.** Histological scoring of DSS treated, and control WT and *Socs2*$^{-/-}$ colons. Data presented as mean ± SEM (n=6). [a]significantly different from DSS treated WT mice $p<0.05$; by unpaired t-test or nonparametric test (Mann-Whitney).

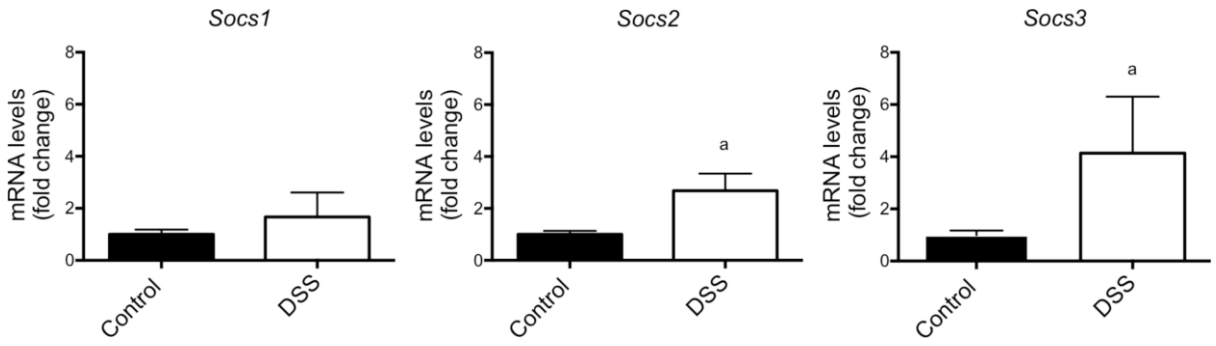


Figure 3. ***Socs*** **mRNA expression in bone from DSS treated WT mice.** *Socs1*, *2*, and *3* mRNA expression levels in tibia from DSS treated WT mice compared to control mice at day 18 (endpoint) of DSS study. Data presented as mean ± SEM. Control group n=6, DSS treated group n=4. [a]significantly different from control samples, $p<0.05$; by nonparametric test (Mann-Whitney).

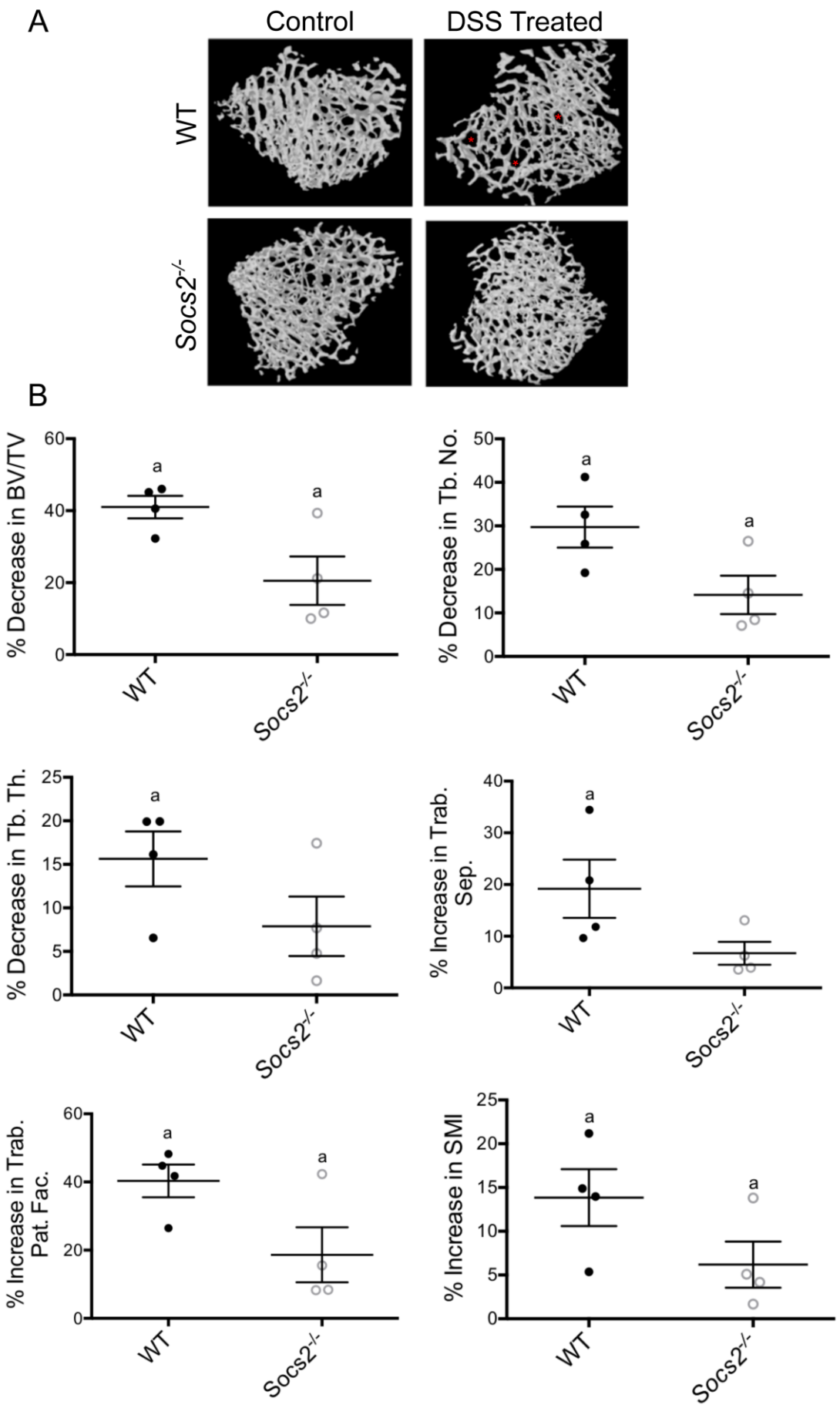


Figure 4. **– Trabecular bone architecture of DSS treated WT and *Socs2*$^{-/-}$ mice. A.** Representative 3D µCT reconstructions showing a less compact trabecular architecture resulting in a more porous structure (*) in DSS treated WT mice compared to WT control mice. A similar alteration in bone architecture was not observed in DSS treated *Socs2* deficient mice. **B.** Percentage change of trabecular parameters in DSS treated mice relative to genotype-matched controls. Data presented as mean ± SEM (n=4). [a]significantly different from genotype-matched control, $p<0.05$; by nonparametric test (Mann-Whitney).

Table 1. Weight and length measurements of WT and *Socs2*$^{-/-}$ mice at day 18 of DSS study.

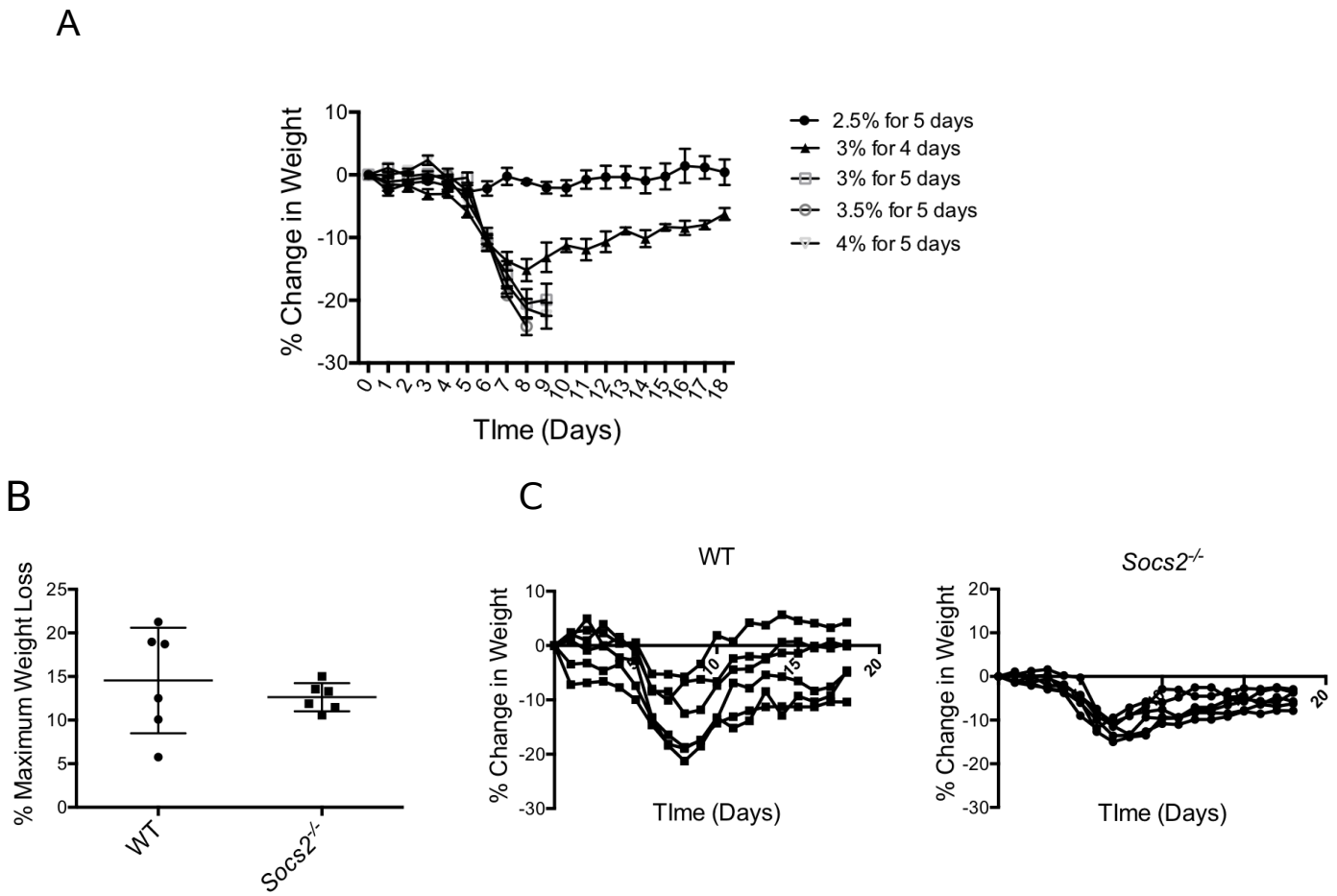
	WT				*Socs2*$^{-/-}$			
	Control		DSS Treated		Control		DSS Treated	
Weight (g)	23.5 ± 0.34		22.6 ± 0.38		32.6 ± 0.38[b]		31.5 ± 0.46	
N to R Length (mm)	88.7 ± 1.11		87.9 ± 1.40		100.5 ± 1.15[b]		103.2 ± 1.35	
Tibia (mm)	17.7 ± 0.14		17.6 ± 0.16		18.7 ± 0.18[a]		18.9 ± 0.10	
Femur (mm)	14.4 ± 0.21		14.4 ± 0.10		15.6 ± 0.08[a]		15.2 ± 0.20	

N to R length = nose to rump length. Data presented as mean ± SEM. All groups n=6 except *Socs2*$^{-/-}$ control femur (n=5). [a]significantly different from WT control mice, $p<0.01$, [b] significantly different from WT control, $p<0.001$; by unpaired t test.

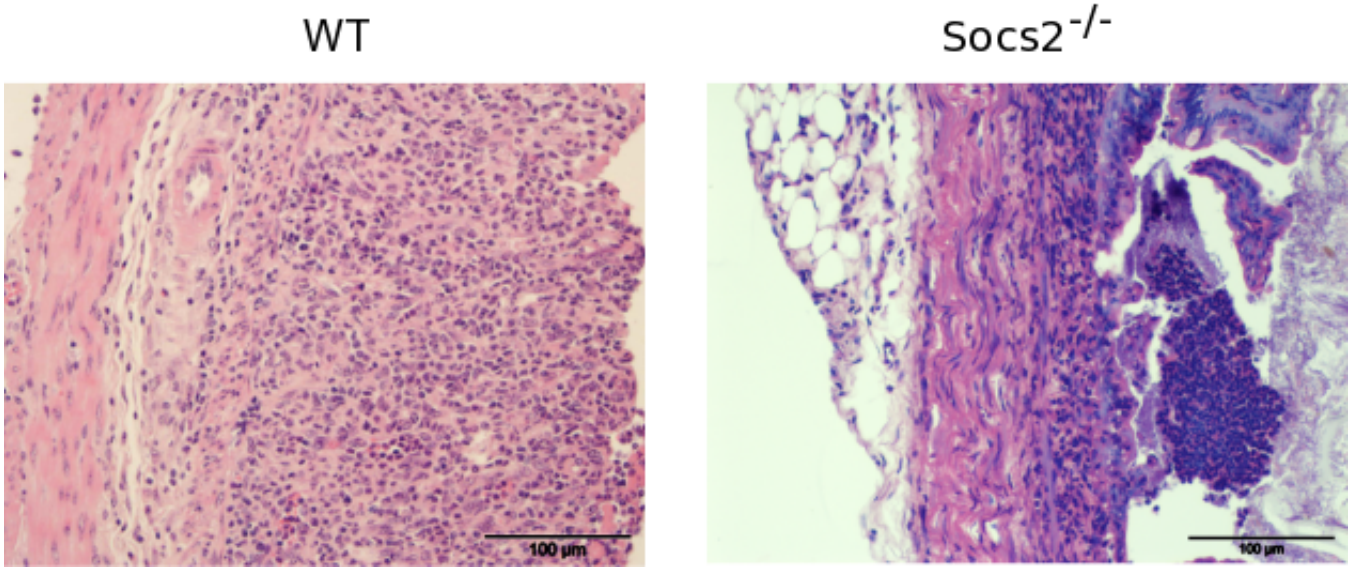
Table 2. Cortical bone parameters of tibia from control and DSS treated WT and *Socs2*$^{-/-}$ mice at day 18 of DSS study.

	WT				*Socs2*$^{-/-}$			
	Control		DSS Treated		Control		DSS Treated	
Tt.Ar (mm²)	0.84 ± 0.001		0.82 ± 0.002		1.03 ± 0.003[a]		1.01 ± 0.003	
Ct.Ar (mm²)	0.54 ± 0.014		0.54 ± 0.012		0.67 ± 0.028[a]		0.65 ± 0.022	
Ma.Ar (mm²)	0.30 ± 0.005		0.28 ± 0.005		0.37 ± 0.009[a]		0.36 ± 0.001	
Ct.Th (mm)	0.23 ± 0.003		0.23 ± 0.004		0.25 ± 0.008[a]		0.25 ± 0.006	

Tt.Ar = total tissue area, Ct.Ar = cortical bone area, Ma.Ar = medullary area, Ct.Th = cortical thickness. Data presented as mean ± SEM (n=4) [a]significantly different from WT control mice, $p<0.05$; by nonparametric test (Mann-Whitney).



Supplementary Figure 1. **DSS concentration and time dependent changes in body-weight. A.** Body weight change of WT mice subjected to varying doses and treatment times of DSS. **B.** Maximum weight loss observed by WT and *Socs2*$^{-/-}$ during DSS experiment. Data presented as mean± SEM (n=6). Data non-significant by unpaired t-test. **C.** Individual weight change observed in WT and *Socs2*$^{-/-}$ mice treated with 3% DSS.



Supplementary Figure 2. **Effects of DSS on mucosal integrity of WT and *Socs2*$^{-/-}$ mice.** Representative image from WT and *Socs2*$^{-/-}$ mice treated with DSS. Images show epithelial degeneration, crypt loss, high levels of mononuclear leukocytes and transmural inflammation.